Prostaglandin-mediated effects in early canine corpus luteum: In vivo effects on vascular and immune factors

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Abstract: Prostaglandins (PGs) are important regulators of the early corpus luteum (CL) in the dog. Whereas, initially, CL is gonadotropin independent, in the second half of its lifespan, hypophyseal support is required. The transition period is marked by decreased availability of PGs, in particular of PGE2. We previously reported that inhibition of COX2/PTGS2 in vivo suppressed luteal production of PGE2, lowered circulating progesterone and negatively affected luteal development. Therefore, bitches were treated with a COX2-specific blocker, firocoxib, for 5, 10, 20 and 30 days after ovulation, leading to suppression of the steroidogenic machinery. Control groups received a placebo for the same periods. Considering the wide range of possible modulatory roles of PGs shown in different organ systems, this follow-up project aimed to understand further possible PG-mediated effects in early canine CL. Thirty-four (34) factors related predominantly to vascularization and immune response were screened (mRNAs and proteins) on samples from the above described in vivo study. Most of the effects were observed during the transitional period (days 20 and 30). The inhibition of COX2 diminished the expression of angiopoietin family members ANGPT1, -2, Tie1 and -2 receptors. The expression of endothelin (ET)-1 was increased. Concerning the immune system, increased expression of the pro-inflammatory cytokines, IL1 , IL6 and IL12a, and elevated expression levels of CD4, was observed. Cumulatively, besides its involvement in regulating steroidogenesis, our results indicate a broader role of PGs in the canine CL, including modulation of angiogenesis, vascular stabilization and local immunomodulation. Possible cross-species translational effects are strongly implied.

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Prostaglandin-mediated effects in early canine corpus luteum: *In vivo* effects on vascular and immune factors

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ABSTRACT

Prostaglandins (PGs) are important regulators of the early corpus luteum (CL) in the dog. Whereas, initially, CL is gonadotropin independent, in the second half of its lifespan, hypophyseal support is required. The transition period is marked by decreased availability of PGs, in particular of PGE2. We previously reported that inhibition of COX2/PTGS2 *in vivo* suppressed luteal production of PGE2, lowered circulating progesterone and negatively affected luteal development. Therefore, bitches were treated with a COX2-specific blocker, firocoxib, for 5, 10, 20 and 30 days after ovulation, leading to suppression of the steroidogenic machinery. Control groups received a placebo for the same periods. Considering the wide range of possible modulatory roles of PGs shown in different organ systems, this follow-up project aimed to understand further possible PG-mediated effects in early canine CL. Thirty-four (34) factors related predominantly to vascularization and immune response were screened (mRNAs and proteins) on samples from the above described *in vivo* study. Most of the effects were observed during the transitional period (days 20 and 30). The inhibition of COX2 diminished the expression of angiopoietin family members ANGPT1, -2, Tie1 and -2 receptors. The expression of endothelin (ET)-1 was increased. Concerning the immune system, increased expression of the pro-inflammatory cytokines, IL1β, IL6 and IL12a, and elevated expression levels of CD4, was observed. Cumulatively, besides its involvement in regulating steroidogenesis, our results indicate a broader role of PGs in the canine CL, including modulation of angiogenesis, vascular stabilization and local immunomodulation. Possible cross-species translational effects are strongly implied.

1. Introduction

The corpus luteum (CL) is a temporary endocrine gland which, by producing progesterone (P4), exerts important roles in the reproduction of mammals. It is formed from the remnants of the ruptured follicle and grows rapidly to ensure an adequate steroid supply for the establishment and maintenance of pregnancy. This rapid development and steroidogenic activity require the support of rapidly developed and dense vascularization [1,2]. Also, immune system-derived factors appear to have an important role in CL formation and function [3]. Prostaglandins (PGs) are among the most prominent regulators of CL function across mammalian species, with PGE2 and PGF2α typically playing opposing roles. Thus, the luteotropic function of luteal PGE2 is mostly opposed to the luteolytic effects of PGF2α. The function of PGE2 is mostly concerned with the cAMP/PKA-mediated stimulation of STAR (steroidogenic acute regulatory protein) expression and function [4,5]. Interestingly, however, some other effects of PGE2 in the CL have been described lately, e.g., with regard to vascularization. Accordingly, e.g., in the pig, PGE2 increases the secretion of luteal VEGF [6].

In the domestic dog, circulating progesterone (P4) originates in the CL, both in pregnant and non-pregnant bitches, thereby acquiring a central role in the regulation of diestrus/pregnancy [7]. When compared with other domestic animal species, canine luteal physiology appears quite peculiar. Thus, in contrast to livestock, a uterine luteolysin (PGF2α) does not exist in non-pregnant bitches, as clearly shown in hysterectomized females which maintain their normal ovarian

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function [8]. Similarly, the presence of an intraluteal luteolytic principle can be ruled out, as there is no luteolysis in the absence of pregnancy in the dog. The intraluteal levels of PGF2α are low [9,10], but the respective receptor (FP/PTGFR) appears, however, to be constitutively expressed [9,11], reaching its highest abundance in the second half of diestrus, and rendering the canine CL receptive to exogenously applied PGF2α. Consequently, the lack of luteolysis in non-pregnant, cyclic bitches results in a prolonged pseudopregnancy, frequently lasting longer than pregnancy itself [4,8]. With this, cumulatively, the luteal regression in non-pregnant dogs appears to be a long-lasting process of structural remodelling and functional degeneration, devoid of an active luteolytic principle [5]. Conversely, in pregnant dogs, the cessation of luteal function is associated with an increased presence of utero-placental PGF2α in the maternal circulation, strongly implying its luteolytic function [7,12].

With regard to species-specific regulatory mechanisms, maintenance of the mature canine CL depends strongly on hypophyseal hormones, with prolactin (PRL) acting as the predominant luteotropic factor during the second half of diestrus, starting around 4 weeks after ovulation [13–15]. During earlier stages of the luteal phase, hypophyseal support does not seem necessary [14] and, at least in part, the CL is gonadotropin independent. At this time, increased expression of luteal cyclooxygenase 2 (COX2/PTGS2), as well as of PGE2 synthase (PTGES) and two cAMP/PKA-mediating PGE2 receptors (EP2/PTGER2 and EP4/PTGER4) in luteal cells, suggests the importance of PGs and in particular of PGE2 as autocrine/paracrine regulators of early CL function in the dog [16,17]. Supporting this idea, *in vitro* stimulation of early canine luteal cells with PGE2 increases the expression of STAR protein and potentiates P4 synthesis [4]. In our previously published studies, the luteotropic role of PGs in the early canine CL was further substantiated *in vivo* [10,18]. Thus, bitches treated with a specific inhibitor of COX2/PTGS2, firocoxib, for up to 30 days after ovulation, exhibited lower levels of intra-luteal PGE2, accompanied by downregulated expression of PTGES and steroidogenesis-linked factors (STAR and 3βHSD), and lowered circulating levels of P4 [10,18]. Interestingly, in a parallel *in vitro* experiment, it was shown that PGE2 positively regulates expression of the prolactin receptor (PRLR) in canine lutein cells. With that previous study [10], a causality has been indicated between PTGS2/COX2 function and the PTGES-dependent synthesis of PGE2, and luteal P4 synthesis in the dog.

As mentioned elsewhere, the development and activity of CL rely on rapid development of a dense vascular network. Accordingly, also in the dog, the luteal expression of VEGFA and VEGFR1 is upregulated in early CL of pregnant and non-pregnant bitches [19,20]. Furthermore, vasoactive factors from the endothelins family are present in the canine CL and are expressed in a time-dependent manner throughout the CL life span [21]. Thus, the early luteal phase is characterized by elevated levels of endothelin 2 (ET2), endothelin receptor B (ETB) and the endothelin converting enzyme 1 (ECE1) that regulates the availability of endothelins [21]. It is noteworthy in this context that, in one of our previous studies, PGE2 was shown to upregulate *in vitro* luteal expression of the vasodilatory endothelin receptor B (ETB) in the dog [21]. The effects of PGE2 on the expression of VEGFA and the endothelin-system have, however, never been assessed *in vivo*. The functionality of the vasculature appears to be also affected during prepartum luteolysis, as implied by the increased expression of vasoconstrictive endothelin A receptor (ETA) [21]. No such effects could be observed for the CL of non-pregnant dogs during late luteal regression [21,22].

With regard to the immune system, apart from its indicated involvement in termination of luteal function in pregnant dogs [22], the development of the CL is also associated with increased activity of immune system-derived factors. This is indicated by the increased infiltration of immune cells [23], as well as increased expression of different chemokines (e.g., tumor necrosis factor alpha; TNFα [24]). Accordingly, increased numbers of CD4, CD8 and endoglin (END) positive cells were observed in the CL of pregnant and non-pregnant cycles [23,24] during luteal formation. Additionally, the expression of several cytokines has been confirmed in canine luteal tissue, e.g., interleukin (IL) 8, IL10, IL12a, TNFα or transforming growth factor 1 beta (TGF1β) [24–26]. Nevertheless, the exact role of these factors in the development and maintenance of the canine CL remains to be elucidated.

In this context, adding to the possible roles of PGE2 in maintaining tissue homeostasis, both pro- and anti-inflammatory mechanisms have been described for this prostaglandin in different tissues [27,28]. In the reproductive system, PGE2 was shown to modulate immune activity in a suppressive manner in the human uterus [29]. These effects seem to support feto-maternal immune tolerance. In other tissues, e.g., the lung, PGE2-induced immunomodulation is linked to control of inflammation and limitation of damage during prolonged immune responses [27,30]. However, to the best of our knowledge, nothing is known about immunomodulatory effects that PGE2 and other PGs could exert in the early canine CL.

Here, taking into account the so far known effects exerted by PGs upon different regulatory systems *in vitro*, and the effects resulting from firocoxib treatment and, thereby, the suppression of PGs function *in vivo*, we hypothesized that PGs could also have other biological effects in the early canine CL. Therefore, using the tissue material from our above mentioned previous studies [10,18], we investigated the possible impact of blocking COX2/PTGS2 on vascular and immune functions during establishment and development of the canine CL. The expression of 34 different potential target genes known for their involvement in regulating CL function was screened.

2. Materials and methods

2.1. Tissue samples

This is a follow up project utilizing tissue samples obtained in our previously described *in vivo* study [10,18], approved by the responsible ethics committee of the University of Warmia and Mazury in Olsztyn, Poland (permit 54/2008).

In brief, middle-sized mixed breed bitches aged 2 to 7 years were observed for the onset of spontaneous estrus by vaginal cytology and P4 assay. The day of ovulation (Day 0) was considered the day when circulating P4 concentrations for the first time exceeded 5 ng/ml. The animals were then randomly assigned (simple randomization) to four treatment groups and five control groups. Bitches from treated groups received, orally, 10 mg/kg body weight per day (twice the recommended dose) of firocoxib (Previcox®, Merial Ltd), a COX2/PTGS2 specific inhibitor, for 5 (n = 4), 10 (n = 4), 20 (n = 3) and 30 (n = 5) days. Animals from control groups received a placebo for 0 (n = 5), 5 (n = 5), 10 (n = 4), 20 (n = 3) and 30 (n = 4) days. With this, samples used in our study derive from different stages of early luteal development in the dog, i.e., developing gonadotropin-independent CL (days 5 and 10), mature CL in transition between gonadotropin independency and dependency (day 20) and mature gonadotropin-dependent CL (day 30) [5,13–15]. This allowed further analysis of the effects of time on the expression of the studied target genes. On the last day of treatment, animals were ovariohysterectomized for collection of ovaries containing CLs. The corresponding P4 levels are presented in [10,18]. Ovaries were then immediately trimmed of surrounding connective tissue and divided into two parts. CLs from one part were preserved for mRNA analysis, being placed in RNAlater (Ambion Biotechnology GmbH, Wiesbaden, Germany) for 24 h at 4 °C and then stored in −80 °C until further use. The second half of the ovary was fixed in 10% phosphate-buffered formalin for 24 h at 4 °C and then paraffin embedded using standard methodology.

2.2. Total RNA extraction, high capacity reverse transcription (RT) and pre-amplification of cDNA

Extraction of total RNA from all samples was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. RNA concentration and purity were measured with a Nano Drop 2000C spectrophotometer (Thermo Fisher Scientific AG, Reinach, Switzerland).

Samples were diluted and a total amount of 10 ng of RNA was used from each sample. To eliminate possible genomic DNA contamination, all samples were treated with the RQ1 RNase-free DNase kit (Promega, Dübendorf, Switzerland) following the manufacturer's protocol. Reverse transcription (RT) was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific, Foster City, CA, USA), following the supplier's protocol, obtaining cDNA in a final volume of 20 µl. Finally, the amount of cDNA was amplified with the TaqMan® PreAmp Master Mix Kit (Applied Biosystems). For this, pooling of TaqMan assays for all selected target genes and reference genes was prepared in a 250 µl mix containing: 180 nM of each forward and reverse primer and 50 nM of probe for commercially available TaqMan assays, and 15 nM of each forward and reverse primer and 20 nM of each probe for self-designed TaqMan systems. For subsequent amplification, 12.5 µl of the previously prepared cDNA from each sample was mixed with 25 µl of 2x TaqMan Preamp Master Mix and 12.5 µl of the pooled TaqMan assays mix. Enzymes were activated at 95 °C for 10 min and then samples were amplified through 14 cycles of 95 °C for 15 s and 60 °C for 4 min, each. All reactions were performed in an Eppendorf Mastercycler (Vaudax-Eppendorf AG, Basel, Switzerland).

2.3. Semi-quantitative real-time TaqMan PCR (qPCR) and data evaluation

Semi-quantification of luteal expression of 34 selected target genes was assessed by real-time (TaqMan) PCR.

For each sample and target gene, 25 µl of reaction mixture was prepared, containing 200 nM TaqMan Probe, 300 nM of each primer, 12.5 µl Fast Start Universal Probe Master (ROX®) (Roche Diagnostics, Mannheim, Germany) and 5 µl of pre-amplified cDNA obtained as described above. All reactions were run in duplicates in 96-well optical plates. Autoclaved water instead of cDNA and the minus-RT control were run as negative controls. A detailed description of primers, TaqMan probes and predesigned (i.e., commercially available) TaqMan systems, as well as all gene names and their corresponding abbreviations are presented in Suppl. File 1. Self-designed primers and 6-carboxyfluorescein (6-FAM) and 6-carbocytetramethylrhodamine (TAMRA) labelled probes were ordered from Microsynth (Balgach, Switzerland). Commercially-available TaqMan systems were obtained from Applied Biosystems. Efficiency values of the PCR reactions were validated to ensure approximately 100%. For this, the CT slope method was performed, using as the template cDNA obtained from CL of two different stages and each diluted in a 10-fold series, according to the instructions of the manufacturer and as previously described [31]. Real-time PCR reactions were run in an automated ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Reactions were activated at 95 °C for 10 min, and then samples were amplified in 40 cycles comprising of: 95 °C for 15 s and 60 °C for 60 s, each. Results were quantified with the comparative CT (ΔΔCT) method according to the protocol provided by the manufacturer of ABI PRISM 7500 and as described previously [16,31]. The expression of the reference genes was used for normalization of target gene expression and the lowest expression for each target gene was used as a calibrator. As for reference genes, in pilot experiments, three genes (*GAPDH*, *β-ACTIN* and *CYCLOPHILIN A*) were initially evaluated, based on their expression in all samples included in the study. Then, evaluation of reference gene stability was performed using two programs: geNorm and NormFinder [32,33]. Finally, *GAPDH* was selected as the most stable gene, compared with *β-ACTIN* and *CYCLOPHILIN A*, and was used as the reference gene for the ΔΔCT evaluation.

To evaluate the effects of treatment on the expression of target genes, an unpaired, two-tailed Student's *t*-test was used, comparing treatment group with control group, from each time point (day). Additionally, time-related changes in the expression of all target genes in control animals were evaluated using Kruskal-Wallis (non-parametric ANOVA) followed by Dunn's test. All numerical results for relative gene expression were logarithmically transformed and are presented as geometric means (Xg) +/- geometric standard deviation (SD). All statistical tests were performed with GraphPad3 (GraphPad Software Inc., San Diego, CA, USA); $P < 0.05$ was considered statistically significant.

2.4. Immunohistochemical staining

Depending on the availability of canine species-specific and/or cross-reacting antibodies, immunohistochemistry (IHC) was performed for those factors that showed a significant response to treatment at the mRNA level. Thus, staining was performed against members of the ANGPT system (ANGPT1, ANGPT2, TIE1 and TIE2), ET1 and CD4; more details are listed below. Antibodies targeted against IL1β (NB600-633, Novus Biologicals LLC, Littleton, CO, USA) and IL12a (Orb256618, Biorbyt Ltd., Cambridge, UK) were also tested, but failed to produce reliable staining. No commercially-available antibody against IL6 with described/predicted cross-reactivity for the canine species was found.

The standard, indirect immunoperoxidase staining method was used for IHC, following our previously published protocol with canine tissues [16]. Briefly, formalin-fixed and paraffin-embedded luteal tissues were cut on a microtome (2–3 µm thick sections), transferred to SuperFrost microscope slides (Menzel-Glaeser, Braunschweig, Germany) and dried overnight at 37 °C. At least 5–6 slides were analysed from each animal. The tissue slices were then deparaffinized with xylene and rehydrated in a graded ethanol series, ending with tap water. Antigen retrieval was obtained by microwave heating in citrate buffer (pH = 6) for three cycles of 5 min at 600 W. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. Slides were washed in IHC buffer/0.3% Triton X pH 7.2–7.4 (0.8 mM Na_2HPO_4, 1.74 mM KH_2PO_4, 2.68 mM KCl, 137 mM NaCl), and incubated in 10% normal horse serum (for TIE2, ET1 and CD4), or 10% normal goat serum (for ANGPT1, ANGPT2 and TIE1), for 20 min at ambient temperature to reduce non-specific binding. Additional blocking with 1.5% bovine serum albumin was performed for 5 min for CD4. Finally, samples were incubated overnight at 4 °C with the following primary antibodies at respective dilutions: anti-ANGPT1 (PAA008Ca01, Cloud-clone Corp., Houston, TX, USA), 1:50; anti-ANGPT2 (TA343276, OriGene Technologies Inc., Rockville, MD, USA), 1:400; anti-TIE1 (sc-9025, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), 1:100; anti-TIE2 (sc-31268, Santa Cruz Biotechnology Inc.) 1:200; anti-ET1 (SIGMA-E166, Sigma-Aldrich, St. Louis, MO, USA), 1:600; anti-CD4 (GTX84720, GeneTex, Irvine, CA, USA), 1:600. Afterwards, samples were incubated in secondary antibody diluted 1:100 (BA-100 biotinylated goat anti-rabbit IgG for ANGPT1, ANGPT2 and TIE1; BA-9500 biotinylated horse anti-goat IgG for TIE2; BA-2000 biotinylated horse anti-mouse IgG for ET1 and CD4; all from Vector Laboratories Inc., Burlingame, CA, USA) for 30 min at ambient temperature, followed by incubation with streptavidin-peroxidase Vectastain ABC kit (Vector Laboratories Inc.) for 30 min at room temperature. Signals were developed with the Liquid DAB + substrate kit (Dako Schweiz AG, Baar, Switzerland). For each antibody, all groups were simultaneously stained using the same development time. Slides were counterstained with hematoxylin, dehydrated in a graded ethanol series and mounted in Histokit (Assistant, Osterode, Germany). For negative controls, sections were incubated with non-immune IgG instead of primary antibodies, from the same species and at the same concentration as the primary antibody (isotype control): rabbit IgG (I-1000) for ANGPT1, ANGPT2 and TIE1, goat IgG (I-5000) for TIE2 and mouse IgG (I-2000) for ET1, all from Vector

Laboratories Inc., and mouse IgG1 for CD4 (ABIN376357, Antibodies-Online GmbH, Aachen, Germany). An additional control was comprised of sections omitting the primary antibody.

Slides were evaluated under a Leica DMRXE light microscope equipped with a Leica DFC425 camera (Leica Microsystems, Wetzlar, Germany). IHC slides were assessed qualitatively by two independent researchers for localization of the respective target proteins and representative pictures were taken. Morphological identification of cellular components within the CL, including immune cell differentiation, was based on available histologic literature [34,35], as well as on information available from the canine literature, and on the herein observed luteal distribution of CD4 expressing cells that corroborates previously published findings [5,23,24,36]. In particular, the localization of CD4 positive signals could be predicted by their typical cellular localization, which was described previously for the dog as being localized in monocytes and macrophages [23,24], thereby serving as a positive internal control.

3. Results

3.1. Semi-quantification of target gene expression

The expression of almost all target genes was detectable in all tissue material available. The only exception was for endothelin 3 (*ET3*), whose expression was frequently below the detection limit, preventing quantitative assessment of its expression. Also, a relatively high individual variability in the expression of some of the studied factors was observed. Despite that, as presented below, treatment and/or time-related changes in the expression of several genes were observed (Fig. 1–3 and Suppl. File 2). On the other hand, no statistically significant changes ($P > 0.05$) were observed for the following genes, neither in their responses to treatment nor during the experimental time-course: *VEGFA*, *ETA*, *MHCII*, *CD8*, *IL8*, *TGF1β*, *TNFR1* and *GATA4* (Suppl. File 3).

3.2. Effects of treatment on target gene expression

Of the 33 detectable factors, effects of Previcox® treatment on mRNA expression were observed on 11: the angiopoietin (ANGPT)-family members (*ANGPT1*, *ANGPT2*, *TIE1* and *TIE2*), as well as *ET1*, *CD4*, *IL1β*, *IL6*, *IL12a*, *cJUN* and *CCNA2*. Thus, *ANGPT1* expression was significantly decreased on day 20 in the treated group ($P < 0.05$, Fig. 1A) while *ANGPT2* ($P < 0.0001$, Fig. 1B), *TIE1* ($P < 0.04$, Fig. 1C) and *TIE2* ($P < 0.04$, Fig. 1D) were decreased on day 30 after ovulation, compared with placebo-treated controls. Conversely, *ET1* expression was increased by treatment on day 20 ($P < 0.03$, Fig. 2A). As for immune factors, whereas the expression of *CD4* was increased on day 30 ($P < 0.03$, Fig. 2B) by the treatment, mRNA levels of *IL1β* ($P < 0.04$, Fig. 2C), *IL12a* ($P < 0.002$, Fig. 2D) and of *IL6* ($P < 0.02$, Fig. 2G) were elevated on day 20. Finally, the expression of *cJUN* ($P < 0.01$, Fig. 3A) and *CCNA2* ($P < 0.01$, Fig. 3B) was also increased at day 20 after ovulation in CL of treated animals. The expression of some of the factors varied greatly in CL of treated animals, e.g., both estrogen receptors, *ERα/ESR1*, and in particular *ERβ/ESR2* or *TNFα*, but their expression levels were not significantly affected by the treatment ($P > 0.05$) (Suppl. File 2).

At the protein level, ANGPT1 was detected in endothelial cells and *tunica media* and pericytes of blood vessels (Fig. 1E). Whereas ANGPT2 was clearly detectable in luteal cells (Fig. 1F), only weak or no signals were detected for ANGPT1 in these cells (Fig. 1E). Additionally, signals for both angiopoietins were detected in interstitial cells identified as luteal macrophages. The expression of ANGPTs in the canine luteal macrophages was verified previously on consecutive sections stained for MHCII/ANGPT2 [36] in the CL of pregnancy. As for angiopoietin receptors, signals for TIE1 were observed in *tunica intima* (endothelial cells), *tunica media* and pericytes of vessels, macrophages and in luteal cells (Fig. 1G), while TIE2 was predominantly localized in vascular endothelial cells and *tunica media*/pericytes of vessels (Fig. 1H). The only member of the endothelin family affected by the treatment, ET1, was localized in luteal cells (Fig. 2E). Finally, CD4-positive cells were identified as monocytes/macrophages (Fig. 2F). Also, this localization pattern of CD4 in immune cells of canine CL was verified previously [24]. In general, the effects of treatment observed at the IHC level appeared to mirror their expression levels observed by qPCR. This was particularly obvious for ANGPT2 at day 30 and for ET1 at day 20. An apparent increase in the number of CD4-positive cells present in CL was observed, mainly in regions surrounding blood vessels.

3.3. Time-related changes in gene expression

Time-related changes in target gene expression were assessed in all control groups from the time of ovulation through early diestrus. During the observation period, the expression pattern of several factors affected by the treatment, *ANGPT2*, *TIE1*, *TIE2, CD4* and *CCNA2*, was time-dependent ($P = 0.0015$, $P = 0.0001$, $P = 0.009$, $P = 0.01$, and $P = 0.001$, respectively) (Figs. 1–3).

Over time, expression of *ANGPT2* and of its receptors, *TIE1* and *-2*, increased. The expression of *ANGPT2* and *TIE1* was significantly higher on days 20 and 30 than during early CL formation ($P < 0.05$, Fig. 1B and C), while the expression of *TIE2* was significantly higher on day 20 compared with the day of ovulation ($P < 0.05$, Fig. 1D). *CD4* expression was more stable in early luteal stages, but it was significantly higher at day 20 than day 30 after ovulation, when its expression was significantly downregulated ($P < 0.01$, Fig. 2B). Finally, representation of the cell cycle regulator *CCNA2* was relatively higher in early CL, showing the highest mRNA levels at day 5 and decreasing significantly by day 20 after ovulation ($P < 0.01$, Fig. 3B).

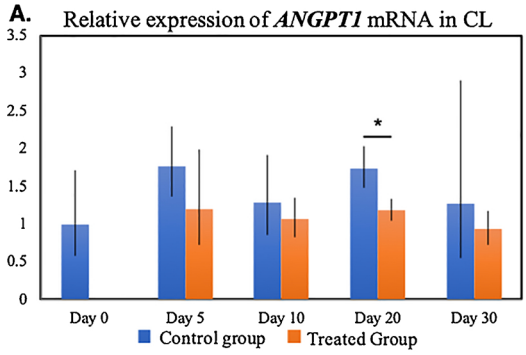
Time-related effects in gene expression were also observed in several target genes that were not affected by the treatment: *ETB* ($P = 0.005$), *ET2* ($P = 0.03$), *ECE1* ($P = 0.03$), *eNOS* ($P = 0.02$), *iNOS* ($P = 0.02$), *ERα/ESR1* ($P = 0.01$), *ERβ/ESR2* ($P = 0.001$), *YY1* ($P = 0.002$), *SF1* ($P = 0.004$), *GATA6* ($P = 0.002$), *END* ($P = 0.01$), *IL10* ($P = 0.03$), *TNFα* ($P = 0.008$) and *TNFR2* ($P = 0.01$) (Suppl. File 2). Additionally, summary of the respective results is presented in Table 1.

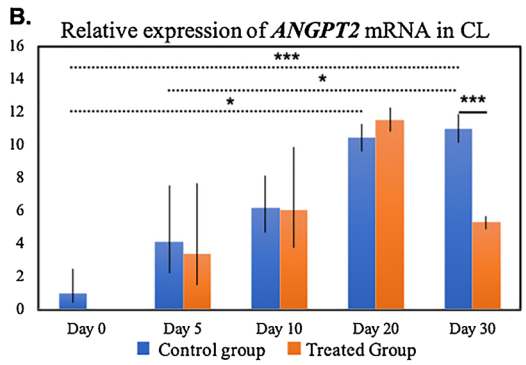
The expression of *ETB* increased rapidly after ovulation, being significantly lower at the time of ovulation than at any other evaluated time points thereafter ($P < 0.05$, Table 1, Suppl. File 2A). In contrast, *ET2* decreased after ovulation, reaching its lowest expression on day 10 ($P < 0.05$, Table 1, Suppl. File 2B). Finally, the levels of mRNA encoding for *ECE1* showed significantly increased levels on days 20 and 30 compared with the day of ovulation ($P < 0.01$) and day 10 ($P < 0.05$, Table 1, Suppl. File 2C). Functionally related to the endothelin-system, expression of *eNOS* (endothelial nitric oxide synthase) and *iNOS* (inducible NOS) was lowest on the day of ovulation, while *eNOS* expression increased significantly towards day 20 ($P < 0.05$, Table 1, Suppl. File 2D), and on days 20 and 30 for *iNOS* ($P < 0.05$, Table 1, Suppl. File 2E). The expression of *ERα/ESR1* increased significantly between the day of ovulation and day 30 ($P < 0.05$ for *ERα*), whereas *ERβ/ESR2* expression was significantly higher on day 30 than on day 10 after ovulation ($P < 0.01$) (Table 1, Suppl. File 2F and G). The levels of mRNA encoding for the transcriptional factors and known regulators of STAR expression, *YY1* and *SF1*, increased significantly after ovulation, with the highest expression on day 20 for *YY1* ($P < 0.01$, Table 1, Suppl. File 2H), and on days 20 and 30 for *SF1* ($P < 0.05$, Table 1, Suppl. File 2I). In contrast, *GATA6* exhibited significantly higher expression on day 5 than on days 20 and 30 ($P < 0.01$ and $P < 0.05$, respectively, Table 1, Suppl. File 2J). As for the immune factors, *END* mRNA levels increased significantly over time, from the day of ovulation towards day 30 ($P < 0.01$, Table 1, Suppl. File 2K). The opposite expression pattern was observed for *IL10*, *TNFα* and its receptor *TNFR2* which showed the highest mRNA abundance on day 5

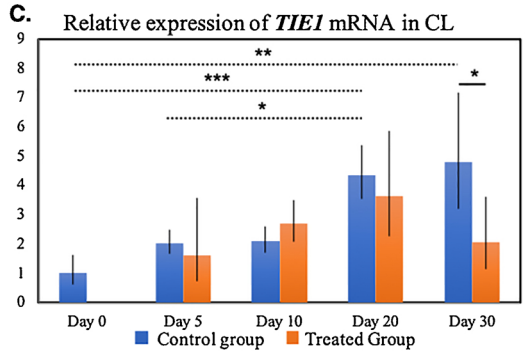
after ovulation, decreasing significantly towards either day 20 (*TNFα*, $P < 0.05$, Table 1, Suppl. File 2M) or day 30 (*IL10* and *TNFR2*, $P < 0.05$, Table 1, Suppl. File 2L and N, respectively).

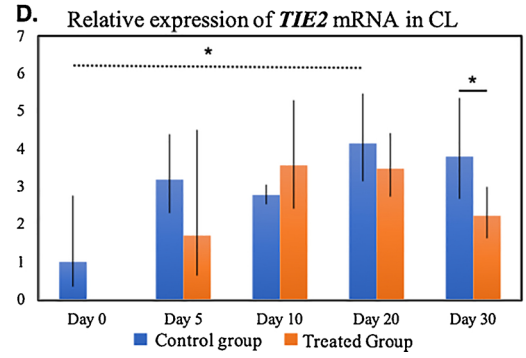
4. Discussion

A plethora of roles has been attributed to PGs in mammals, including those roles less studied with respect to the reproductive tract, such as immunomodulation/suppression or vasodilation and









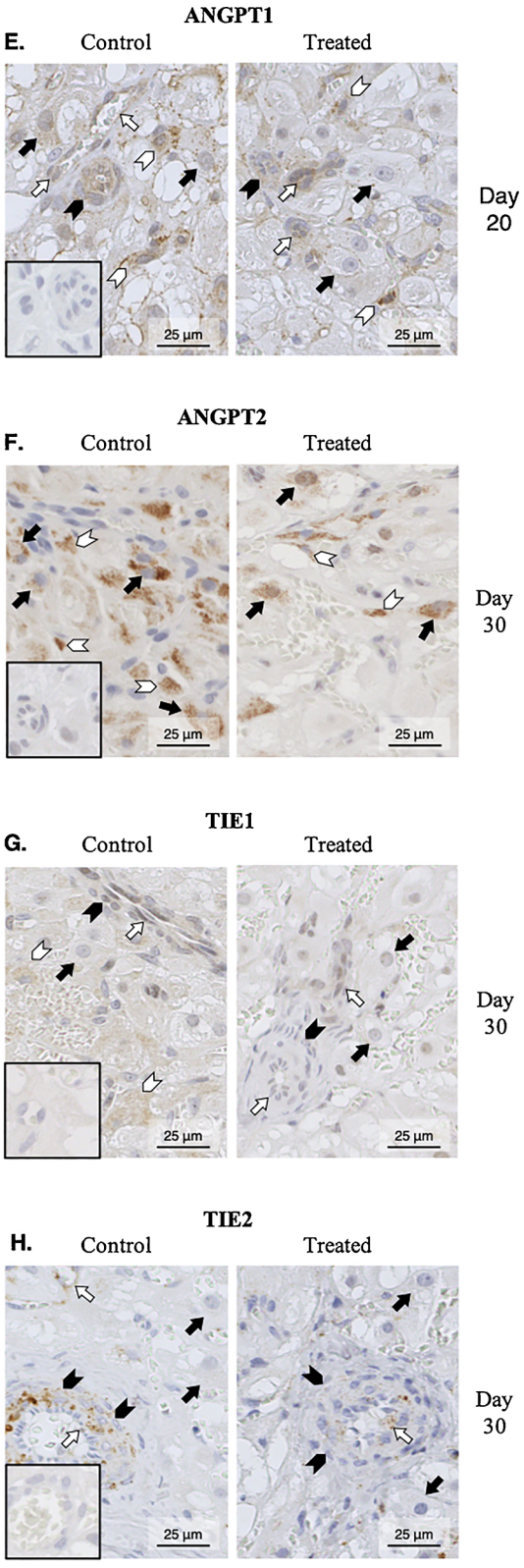


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Fig. 1. Expression of ANGPT1, ANGPT2, TIE1 and TIE2 in luteal tissue of control and Previcox-treated dogs. (A–D) Relative target gene expression as determined by Real Time (TaqMan) PCR (Xg ± SD). The Kruskal–Wallis (non-parametric ANOVA) was applied to test the effects of time on gene expression in all control samples, revealing: $P > 0.05$ for *ANGPT1,* $P = 0.0015$ for *ANGPT2*, $P = 0.0001$ for *TIE1* and $P = 0.009$ for *TIE2*. This, in case of $P < 0.05$, was followed by Dunn's multiple comparison post-test (dotted lines). Student's *t*-test was applied to test the effect of treatment on gene expression (solid lines). Bars with asterisks differ at: * $=P < 0.05$, ** $=P < 0.01$, *** $=P < 0.001$. (E–H) Immunohistochemical localization of respective proteins; representative pictures are shown at selected time points after ovulation. (E) ANGPT1 protein is detected in endothelial cells (open arrows), *tunica media* or pericytes of vessels (solid arrowheads) and macrophages (open arrowheads). Weaker or no signals are detected in luteal cells (solid arrows). (F) ANGPT2 protein is localized predominantly in luteal cells (solid arrows) and luteal macrophages (open arrowheads). (G) TIE1 localizes in endothelial cells (open arrows), *tunica media* or pericytes of vessels (solid arrowheads), macrophages (open arrowheads) and luteal cells (solid arrows). (H) TIE2 stains in endothelial cells (open arrows) and *tunica media* or pericytes (solid arrowheads). Weaker or no signals are detected in luteal cells (solid arrows). No staining is observed in the isotype control (left bottom pictures in E–H, shown at the same magnification).

vasoconstriction. As for the CL of the dog, in particular PGE2 is considered to be among the important regulators in the early, gonadotropin-independent developmental stage. Its function has been associated predominantly with cAMP-dependent regulation of steroidogenesis [5]. Consequently, based on the possible causality implied in our previous studies between COX2/PTGS2 activity and the functionality of PGE2 in Previcox-treated dogs [10,18], here, other potential biological effects exerted by PGs in the canine CL were investigated. The main focus was on the vascular and immune systems. Thus, a wide range of factors possibly involved in the regulation of luteal function was screened for their expression during the normal early luteal phase and following application of Previcox. According to our knowledge, some of these factors were thoroughly studied and/or quantitatively assessed for the first time in the early canine CL, e.g., *IL1β*, IL6, *IL12a*, *IL10, eNOS* and *iNOS*. Additionally, the expression of some other factors involved in the establishment and maintenance of CL function was examined, such as *ERα/ESR1* and *ERβ/ESR2*, or transcriptional regulators of *STAR*: *GATA4* and *-6*, *SF1, cJUN* and *YY1*. Despite large individual variations in the expression of most of the investigated factors upon treatment with Previcox (discussed in more detail later), several of them were identified as being strongly affected by PGs withdrawal.

4.1. Vascular factors

Among the vascular factors, the ANGPT system was the most strongly affected by Previcox treatment in our study. This study is the first to show expression of the ANGPT system during CL development in non-pregnant dogs. Its expression in the CL of pregnant dogs was shown recently [36]. Whereas the expression of ANGPT1 was time-dependent, showing strong decrease towards prepartum luteolysis, the expression of ANGPT2 remained highly expressed and clearly detectable over time [36]. Similarly, in the herein presented study, CL formation was associated with increasing mRNA levels of *ANGPT2* and the two receptors, *TIE1*, and *TIE2*. The expression of *ANGPT1* remained more or less stable during the 30 days of luteal development of non-pregnant dogs. Consistent with our previous findings with CL of pregnancy [36], the expression of ANGPT1 was targeted to luteal vessels, whereas ANGPT2 was localized mainly in luteal cells. Also, interstitial cells and cells identified as local macrophages [24,34–36], were identified as a possible source of ANGPTs in the canine CL. Both receptors were predominantly localized in the vascular components of the CL, pointing towards functional interplay of the ANGPT family members in regulating the canine CL. Acting together with VEGFA, and utilizing their tyrosine kinase receptors, TIE1 and TIE2, ANGPTs regulate vascular stability [37–40]. Whereas ANGPT1 promotes vascular stabilization and prevents uncontrolled angiogenesis [37,38], ANGPT2 in the presence of abundant concentrations of VEGFA (e.g., during the early luteal phase) causes destabilization/loosening of blood vessels and supports migration of endothelial cells, enhancing angiogenesis [2,41]. The results of our study indicate that the negative effects of Previcox treatment on expression of ANGPT family members point towards modulatory effects of PGs on luteal angiogenesis and vessel stabilization. Taking into consideration the cellular distribution of ANGPTs and their receptors in Previcox treated animals, we propose that PGs have stimulatory effects on the ANGPT system in different luteal cellular compartments.

Interestingly, the expression of VEGFA was not affected by the treatment in the present study. Thus, the functional connection between locally acting PGs, in particular PGE2, and the VEGF system, established previously for the pig and rat CL [6,42], does not seem to apply in the dog.

Furthermore, adding to the positive effects exerted by PGE2 on ETB expression in isolated canine lutein cells [21], here we observed modulatory effects of PGs withdrawal on ET1. In agreement with our previous study [21], ET1 in the canine CL was localized in lutein cells. Endothelins play important roles in the regulation of several ovarian functions such as vascularization, steroidogenesis, ovulation, folliculogenesis, luteal regression and luteolysis [43,44]. Besides ET1, they are represented by ET2 and ET3, and upon activation by the specific converting enzyme (ECE1), endothelins exert their ETA- and ETB-mediated effects, the latter involving activation of the nitric oxide (NO) pathway [45,46]. Here, although *ETB* expression seemed to be lowered by Previcox treatment, these changes were not statistically significant, most probably due to high individual variations.

Taking into account, however, the above-mentioned effects of PGE2 on ETB expression and the stimulatory effects exerted by PGs suppression on *ET1* expression, it seems that the role of PGs in regulating the expression and function of luteal endothelins is a topic worthy of more attention in future research. In addition, ET1 has been linked to luteolytic processes in several species, e.g., in ruminants, rats and rabbits [47–50]. Similarly, along with the vasoconstrictive ETA, the expression of ET1 was strongly increased in our previous study in which a PGR blocker was applied for pregnancy termination in mid-pregnant dogs [21].

On the other hand, in accordance with our previous findings, CL development was characterized by increased intraluteal levels of *ETB* and *ECE1* [21]. The increased vascularization observed during this time was also mirrored in increasing expression of endoglin (*END*), which is a phenomenon described previously for the CL of both pregnant and non-pregnant dogs [23,24]. Similarly, expression of the respective NO synthases, *eNOS* and *iNOS*, known for their involvement in regulating luteal function in other species, such as cattle or rats [51–53], increased significantly with progression of the luteal phase. In the present study, however, their expression was unaffected by Previcox treatment.

4.2. Immune factors

The early luteal phase in the dog is associated with increased infiltration of CD4- and CD8-positive cells, mostly representing macrophages, monocytes and lymphocytes [23,24]. Their presence and distribution within the CL of pregnant and non-pregnant dogs has been described before [23,24]. Particularly for CD4 expression, we were able to confirm these previous observations in the present study. A new finding from our study was, however, the increased expression of *CD4* in the CL of Previcox treated animals. As for other mediators of the immune response, the pro-inflammatory *IL1β* and *IL12a* did not vary strongly individually nor with regard to the stages of early CL

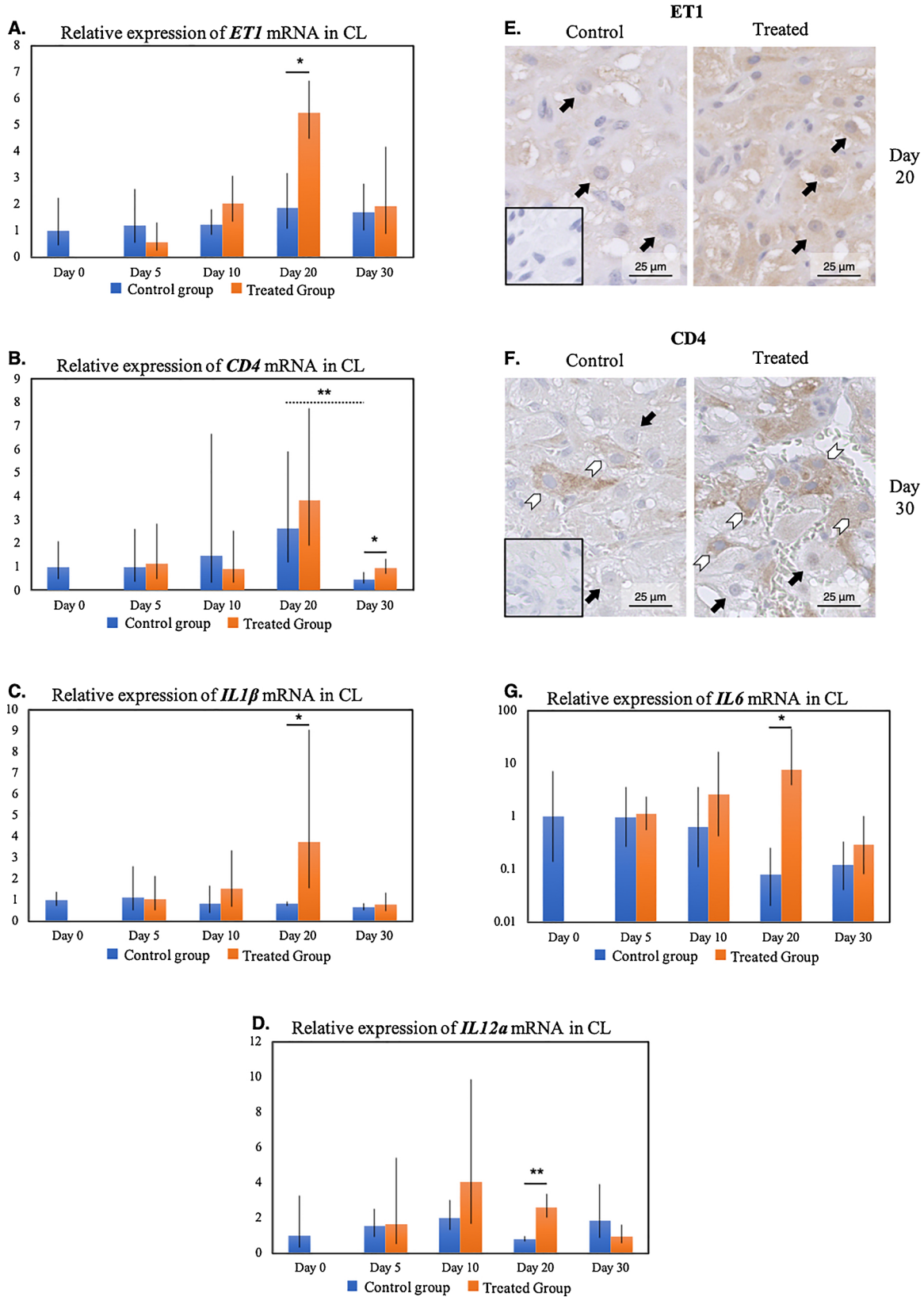

Fig. 2. Expression of ET1, CD4, IL1β, IL6 and IL12a in luteal tissue of control and Previcox-treated dogs. (A–D and G) relative target gene expression as determined by Real Time (TaqMan) PCR (Xg ± SD). The Kruskal-Wallis (non-parametric ANOVA) was applied to test the effects of time on gene expression in all control samples, revealing: $P > 0.05$ for *ET1*, *IL1β*, *IL6* and *IL12a*, and $P = 0.01$ for *CD4*. This, for CD4, was followed by Dunn's multiple comparison post-test (dotted line). Student's *t*-test was applied to test the effect of treatment on gene expression (solid lines). Bars with asterisks differ at: *=$P < 0.05$; **=$P < 0.01$. (E–F) Immunohistochemical localization of ET1 and CD4; representative pictures are shown at selected time points after ovulation. (E) ET1 protein is localized in luteal cells (solid arrows). (F) CD4 protein is localized in monocytes/macrophages (open arrowheads); lutein cells are indicated by solid arrows. No staining is observed in the isotype control (left bottom pictures in E and F, shown at the same magnification).

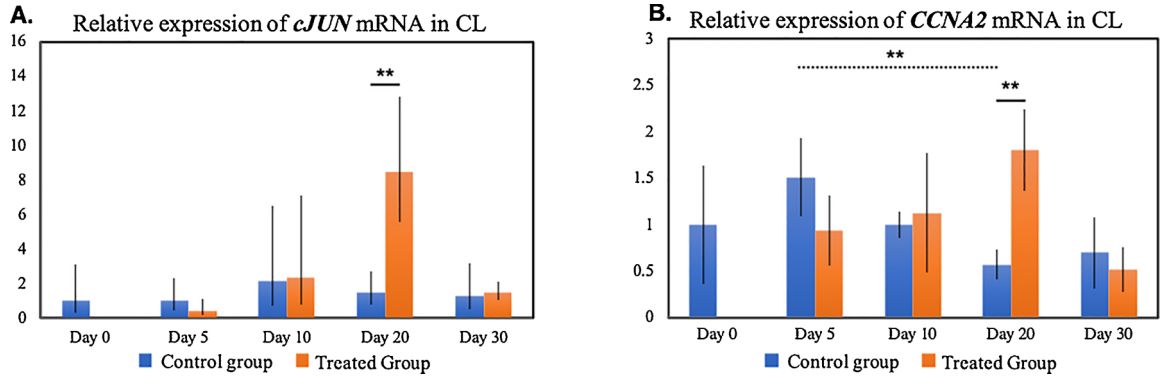

Fig. 3. Expression of *cJUN* and *CCNA2* mRNA in luteal tissue samples of control and Previcox-treated dogs. Relative target gene expression was determined by Real Time (TaqMan) PCR (Xg ± SD). The Kruskal-Wallis (non-parametric ANOVA) was applied to test the effects of time on gene expression in all control samples revealing: $P > 0.05$ for cJun and $P = 0.001$ for CCNA2. In case of CCNA2 it was followed by Dunn's multiple comparison post-test (dotted line in B). Student's *t*-test was applied to test the effect of treatment on gene expression (solid lines). Bars with asterisks differ at: ** = $P < 0.01$.

development. The expression of *IL6* seemed to be lower at day 20 and 30, but also these effects were not statistically significant. With regard to Previcox treatment, like for CD4-expressing cells, the expression of all three pro-inflammatory cytokines (*IL1β*, *IL12a* and *IL6*) was significantly increased. This was, indeed, an interesting discovery from our study as it indicates modulatory, possibly immunosuppressive, effects of PGs in the canine CL. The pro-inflammatory properties of IL1β in the CL of the dog were recently implicated in our transcriptome studies, revealing its increased presence in apoptosis-dominated CL during prepartum luteolysis [22]. Conversely, the elevated expression of *IL10* during early CL development, at day 10 after ovulation, indicates its possible luteotropic function, resembling thereby the situation described previously for human luteal cells, in which IL10 was shown to stimulate P4 production [54].

Finally, whereas the expression of *TNFR1* was not significantly affected in our study, the expression of *TNFα* and *TNFR2* was higher in early developing CL, resembling thereby their expression patterns described previously [24]. The TNF-system was, however, not modulated in response to the suppression of PGs synthesis.

4.3. Estrogen receptors

When expression of the estrogen receptors, *ERα/ESR1* and *ERβ/ESR2*, was examined, similarly to what has been observed for the *PGR* [10], their mRNA levels remained unaffected by treatment with Previcox. Time-related changes in the expression of these two receptors in the canine CL were shown previously [55]. In accordance with our findings, here, the expression of *ERα/ESR1* increased between luteal formation and mid-diestrus. However, and in contrast to the previous report, changes in *ERβ/ESR2* were also found in this study, revealing its increasing expression towards mid-diestrus. This difference could be explained by the high variability in circulating levels of 17β-estradiol (E2) [7], possibly exerting regulatory effects on its own receptors.

4.4. Factors involved in steroidogenesis

Knowing the importance of some key transcriptional factors in regulating STAR expression, we decided to assess the basic capability of luteal PGs to modulate their expression in the CL following the suppression of prostaglandin synthesis. Among the five regulators studied here (*cJUN*, *SF1*, *GATA4*, *GATA6* and *YY1*), only *cJUN* expression was affected by the treatment. The cJUN is a member of the AP1-family of transcriptional factors, and is well known for its involvement in regulating STAR promoter activity [56]. In pregnant dogs, its luteal expression was suppressed towards prepartum luteolysis, mirroring the diminishing STAR expression and circulating P4 levels at that time [22]. Knowing this, it was perplexing to observe increased expression of *cJUN* in response to Previcox treatment at day 20 after ovulation. A similar effect was observed for *CCNA2*, which encodes for cyclin-A2 protein, a positive regulator of the cell cycle [57]. Since, clearly, as observed in our previous studies, blocking COX2/PTGS2 led to a decrease in the size of luteal cell nuclei (indicating diminished transcriptional activity), lower STAR expression and lowered P4 levels [10,18], the upregulation of *cJUN* and *CCNA2* in treated CL appears to result from the presence of some, as yet undefined, compensatory mechanisms in the CL responding to the functional withdrawal of PGs. On the other hand, since the mRNA expression of *cJUN* and *CCNA2* was examined here, it is thus possible that their functional availability is regulated at the protein level or through post-translational modifications, such as phosphorylation [56].

4.5. Conclusions

Interestingly, in our study, most of the effects of Previcox treatment on target gene expression were observed at days 20 and 30 after ovulation. These days fall into the transitional stage between independence and dependence on hypophyseal hormones for CL maintenance [14]. Afterwards, PRL is the main regulator of CL function [58–60]. On the other hand, even though PRL is necessary for CL maintenance, and together with LH its circulating concentrations continuously increase, luteal regression still takes place. At the same time, during progression of the luteal phase, the expression of PRLR in the CL decreases, resembling the course of circulating P4 concentrations [59]. Consequently, in view of our recent findings including the positive effects of PGE2 on PRLR expression in lutein cells [10], and having noted the suppressive effects of Previcox on PRLR expression in the CL, here a new hypothesis is proposed: decreasing support of PGs during the gonadotropin-dependent luteal stage in the dog might lead to lowered PRLR expression and contribute, thereby, to ongoing CL degeneration and diminishing P4 levels.

Thus, indeed, the transition period between the two functional stages of the canine CL appears to be of the utmost importance, marking a change in the regulatory mechanisms of luteal function and the transition between the developing CL, with a high steroidogenic output, and the maintenance luteal stage exhibiting a slow degeneration of the CL accompanied by a progressive decrease in P4 production.

In this context, the results of the present work indicate a modulatory role of PGs that goes beyond the gonadotropin-independent phase, and this despite the observed large variations in expression of several of our target genes. These variations could be due to the limited number of animals available per group and/or individual variations between these animals. On the other hand, however, in particular in the CL of

Table 1
Expression of target genes significantly affected by time. Relative gene expression (RGE) is presented for each control group as geometric mean and geometric standard deviation (± SD). Kruskal–Wallis (non-parametric ANOVA) was applied, followed by Dunn's test. Only comparisons with significant P-value at Dunn's test are represented. $P < 0.05$ was considered statistically significant.

Target	Group	RGE	SD+/SD−	ANOVA p Value	Dunn's test
ETB	Day 0 control	1	10.79/0.92	P = 0.005	d0 vs d5 – P < 0.05 d0 vs d10 – P < 0.05 d0 vs d20 – P < 0.05 d0 vs d30 – P < 0.05
	Day 5 control	26.6	13.82/9.1		
	Day 10 control	42.37	11.28/8.91		
	Day 20 control	35.55	28.66/15.87		
	Day 30 control	17.04	22.34/9.67		
ET2	Day 0 control	5.92	16.57/4.36	P = 0.03	d0 vs d10 – P < 0.05
	Day 5 control	1.66	2.97/1.06		
	Day 10 control	0.99	0.74/0.42		
	Day 20 control	1.88	1.57/0.86		
	Day 30 control	1.86	3.76/1.25		
ECE1	Day 0 control	1	0.77/0.43	P = 0.03	d0 vs d20 – P < 0.01 d0 vs d30 – P < 0.01 d10 vs d20 – P < 0.05 d10 vs d30 – P < 0.05
	Day 5 control	2.04	1.04/0.69		
	Day 10 control	1.45	0.79/0.51		
	Day 20 control	3.17	1.87/1.18		
	Day 30 control	2.88	2.27/1.27		
eNOS	Day 0 control	1	1.07/0.52	P = 0.02	d0 vs d20 – P < 0.05
	Day 5 control	3.1	2.17/1.28		
	Day 10 control	2.16	0.34/0.29		
	Day 20 control	4.61	0.61/0.54		
	Day 30 control	2.94	5.06/1.86		
iNOS	Day 0 control	1	0.91/0.48	P = 0.02	d0 vs d20 – P < 0.05 d0 vs d30 – P < 0.05
	Day 5 control	1.82	1.22/0.73		
	Day 10 control	2.14	0.57/0.45		
	Day 20 control	3.78	2.33/1.44		
	Day 30 control	3.51	1.67/1.13		
ERα/ESR1	Day 0 control	0.99	3.75/0.79	P = 0.01	d0 vs d30 – P < 0.05
	Day 5 control	2.46	1.59/0.97		
	Day 10 control	4.33	4.96/2.31		
	Day 20 control	5.2	1.27/1.02		
	Day 30 control	9.19	4.45/3		
ERβ/ESR2	Day 0 control	1	0.65/0.39	P = 0.001	d10 vs d30 – P < 0.01
	Day 5 control	0.82	0.56/0.33		
	Day 10 control	0.44	0.25/0.16		
	Day 20 control	0.67	0.16/0.13		
	Day 30 control	1.24	0.67/0.44		
YY1	Day 0 control	1	1.4/0.58	P = 0.002	d0 vs d20 – P < 0.01
	Day 5 control	2.39	1.52/0.93		
	Day 10 control	3.2	2.14/1.28		
	Day 20 control	3.43	0.52/0.45		
	Day 30 control	2.92	0.82/1.2		
SF1	Day 0 control	1	1.45/0.59	P = 0.004	d0 vs d20 – P < 0.05 d0 vs d30 – P < 0.05
	Day 5 control	2.11	1.27/0.79		
	Day 10 control	2.59	2.38/1.24		
	Day 20 control	3.08	1.44/0.98		
	Day 30 control	3.03	2.4/1.34		
GATA6	Day 0 control	1.02	0.13/0.12	P = 0.002	d5 vs d20 – P < 0.01 d5 vs d30 – P < 0.05
	Day 5 control	1.14	0.28/0.22		
	Day 10 control	0.91	0.35/0.25		
	Day 20 control	0.68	0.16/0.13		
	Day 30 control	0.61	0.51/0.28		
END	Day 0 control	1	0.58/0.37	P = 0.01	d0 vs d30 – P < 0.01
	Day 5 control	1.7	0.44/0.35		
	Day 10 control	1.16	0.13/0.12		
	Day 20 control	2.21	0.86/0.62		
	Day 30 control	2.32	2.58/1.22		
IL10	Day 0 control	1	2.71/0.73	P = 0.03	d5 vs d30 – P < 0.05
	Day 5 control	3.44	3.97/1.84		
	Day 10 control	2.27	1.5/0.91		
	Day 20 control	0.68	0.18/0.14		
	Day 30 control	0.77	0.1/0.09		

(*continued on next page*)

Table 1 (*continued*)

Target	Group	RGE	SD+/SD−	ANOVA p Value	Dunn's test
TNFα	Day 0 control	1	2.36/0.7	P = 0.008	d5 vs d20 – P < 0.05
	Day 5 control	2.88	1.71/1.08		
	Day 10 control	2.6	3.59/1.51		
	Day 20 control	0.75	1.15/0.46		
	Day 30 control	1.31	0.84/0.51		
TNFR2	Day 0 control	1	1.13/0.53	P = 0.01	d5 vs d30 – P < 0.05
	Day 5 control	2.63	0.87/0.65		
	Day 10 control	1.87	1.02/0.66		
	Day 20 control	1.45	0.54/0.39		
	Day 30 control	0.98	0.27/0.21		

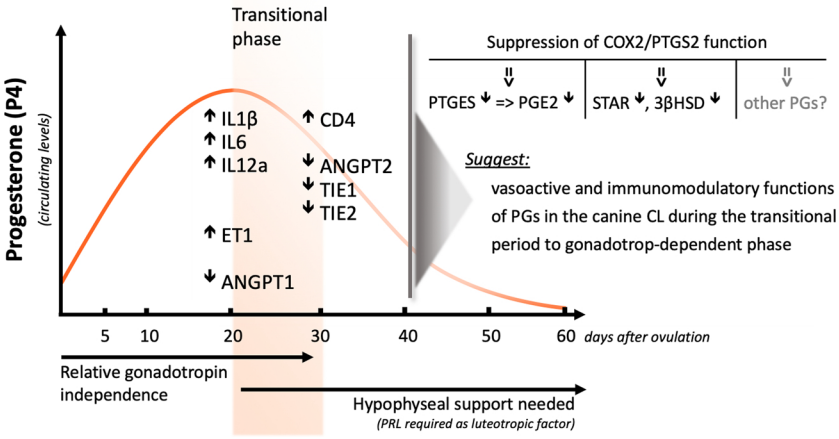

Fig. 4. Schematic representation of effects of functional inhibition of cyclooxygenase 2/prostaglandin synthase 2 (COX2/PTGS2) with Previcox on vascular and immune factors investigated in the present *in vivo* study in the CL of non-pregnant dogs during first 30 days of diestrus. The main findings of the present study are summarized, placing them in perspective with the main features of the canine luteal phase, mainly circulating progesterone (P4) levels and different regulatory phases of the canine CL. Arrows indicate increased (↑) or decreased (↓) expression of different factors in response to inhibition of COX2/PTGS2 activity. When applied in the early luteal phase, Previcox treatment modulates the expression of different factors related to the vascular and immune systems: *ANGPT1*, *ANGPT2*, *TIE1*, *TIE2* (angiopoietin-family, i.e., angiopoietins and their TIE receptors), *ET1* (endothelin 1), interleukins (*IL*)-*1β*, *IL6* and *IL12a* (pro-inflammatory interleukins), *CD4* (cluster of differentiation 4). Together with the previously observed causality between COX2/PTGS2 functionality and luteal PGE2 synthesis by PTGES [10], the decreased expression of several factors involved in regulating CL function in treated dogs suggests a modulatory role of PGs in vascularization and immunity of the canine CL during the transitional phase between independence and dependence on hypophyseal hormones.

Previcox-treated animals, they could possibly also arise from the presence of some compensatory mechanisms, as indicated by the effects of treatment on *cJUN* and *CCNA2* expression. Since the tissue material used for the present study derived from a closed study, we were not able to include additional samples. In the present study, after consulting with the manufacturer for drug safety, double the recommended effective dosage of Previcox was administered to the animals. Despite this higher dosage, pharmacokinetics of firocoxib could reduce its local concentrations at the ovary. This could affect the local effectiveness of the drug, additionally contributing to the obtained variation. Nevertheless, in the context of canine CL physiology, supported by our previous reports [10,59], our observations seem encouraging for further investigation into canine CL regulation.

Finally, supporting our hypothesis, the results presented herein, summarized in Fig. 4, show some potential novel roles of PGs in regulation of the early canine CL, going beyond the P4-stimulatory effects. The involvement of other COX2/PTGS2-derived metabolites on luteal function in the dog remains to be elucidated. Changes induced by inhibition of COX2/PTGS2 in the expression of ANGPT family members (regulators of vascular stability or disruption), seem to indicate a role of PGs in blood vessel stabilization. Also, the increased expression of ET1 in treated animals, as well as of CD4 and pro-inflammatory interleukins, point towards a (immuno)protective role of PGs in the maintenance of CL function.

Having the life span of the CL divided into gonadotropin-dependent and -independent phases, the dog appears to be an interesting model to study mechanisms regulating CL function in a species in which the luteal phase is not affected by possible masking effects of gonadotropins, in particular during early CL development.

Conflict of interests

The authors declare that they have no conflict of interests. All authors read and approved the final version of the manuscript.

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Authors' contribution

MTP was involved in developing the concept of the present study, experimental design, generating data, analysis and interpretation of data and drafting of the manuscript. AG and RN were involved in knowledge transfer, and in the laboratory part of the project, as well as in critical discussion and evaluation of data. TJ was involved in the design of *in vivo* study, tissue collection, knowledge transfer, critical discussion of data and revision of the manuscript. BH was involved in the design of *in vivo* study. BH and AB were involved in knowledge transfer, critical discussion of data and revision of the manuscript. MPK designed and supervised the project, and was involved in interpretation of the data, and drafting and revision of the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.repbio.2019.02.001.

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